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Identification and mutational analysis of the promoter for a spinach chloroplast transfer RNA gene

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A transcription extract from purified spinach chloroplast was used to test chloroplast DNA sequences for their function as promoter elements. Chloroplast tRNA genes are correctly transcribed in the extract by a soluble RNA polymerase, and precursor molecules are processed into mature tRNAs. Transcription of the spinach chloroplast $tRNA_2^{Met}$ gene (*trn*M2) *in vitro* requires 5′ upstream DNA sequences. Deletion of 5′ DNA sequences with exonuclease *Bal*31 was used to establish the 5′ boundary of the promoter region. This boundary is part of a DNA sequence with partial homology to the prokaryotic −35 region. Seventeen base pairs downstream from this sequence a DNA sequence occurs which is homologous to the prokaryotic −10 region. We used synthetic oligonucleotides fused to *trn*M2 5′ deletion mutants to create insertions, deletions and base substitutions in these regions. Internal deletion mutants demonstrated that the −10 promoter element is also required for transcription *in vitro*. The arrangement of DNA sequences recognised by the chloroplast RNA polymerase resembles the prokaryotic promoter organization.

Key words: chloroplast tRNA gene/*in vitro* mutagenesis/*in vitro* transcription/promoter structure

Introduction

Higher plant plastid genes are transcribed by plastid RNA polymerase(s) which are distinct from their nuclear counterparts. Some biochemical properties are known for purified chloroplast RNA polymerases (Bottomley *et al.*, 1971; Smith and Bogorad, 1974; Polya and Jagendorf, 1971; Brandt and Wiessner, 1979; Briat and Mache, 1980; Tewari and Goel, 1983; Wollgiehn, 1982), chloroplast DNA/RNA polymerase complexes (transcriptionally active chromosomes; Hallick *et al.*, 1976; Dron *et al.*, 1979; Briat *et al.*, 1979; Gruissem *et al.*, 1983a; Greenberg *et al.*, 1984a), or for RNA polymerase activities present in recently developed chloroplast *in vitro* transcription systems (Gruissem *et al.*, 1983a, 1983b; Gruissem, 1984; Gruissem and Zurawski, 1984; Link, 1984). Much speculation has occurred in the past regarding the promoter sequences required for transcription initiation of plastid genes by RNA polymerase(s). DNA sequences for several chloroplast genes are now available and comparison of 5′-flanking regions in higher plant chloroplast genomes has revealed DNA sequences with good or partial homology to prokaryotic −35 (tcTTGACat) and −10 (TAtAaT) promoter elements (McIntosh *et al.*, 1980; Zurawski *et al.*, 1981, 1982a, 1982b; Shinozaki and Sugiura, 1982; Krebbers *et al.*, 1982; Deno *et al.*, 1983; Alt *et al.*, 1983; Bohnert *et al.*, 1982; Whitfeld and Bottomley, 1983). These regions, especially when occurring at appropriate spacings proximal to putative or experimentally defined transcription initiation sites, were ascribed as promoter elements in chloroplast DNA (Bohnert *et al.*, 1982; Whitfeld and Bottomley, 1983; Steinmetz *et al.*, 1983; Krebbers *et al.*, 1984; Crouse *et al.*, 1984). The recent development of chloroplast *in vitro* transcription systems has allowed the characterization of chloroplast promoter sequences in more detail (Gruissem *et al.* 1983a, 1983b; Link, 1984; Gruissem and Zurawski, 1984). *Bal*31 deletion of DNA sequences showed that 5′ upstream sequences are required for the expression of chloroplast genes in spinach (Gruissem *et al.*, 1983b; Gruissem and Zurawski, 1984) and mustard (Link, 1984). For a more complete analysis of regulatory and promoter regions for plastid genes, we have used a spinach chloroplast *in vitro* transcription system which supports the correct expression of tRNA and protein coding genes (Gruissem *et al.*, 1983b; Gruissem, 1984; Gruissem and Zurawski, 1984). Here, we show by *in vitro* modification of cloned DNA templates that transcription of at least one spinach chloroplast DNA-encoded gene (*trn*M2) requires 5′ upstream regions for the initiation of transcription. The essential sequences were traced to two sequence blocks which resemble the prokaryotic promoter elements (Pribnow, 1975; Rosenberg and Court, 1979; Siebenlist *et al.*, 1980; Hawley and McClure, 1983). We present evidence that mutations in these regions can alter the transcription rate and that a specific arrangement of DNA sequences is required for maximal transcription.

Results and Discussion

The spinach chloroplast trnM2 transcription unit

The spinach chloroplast *trn*M2 transcriptional unit occurs between the *atp*BE and *trn*V1 transcriptional units in the arrangement 3′ *atp*BE-208 bp spacer-3′ *trn*M2 (73 bp)-166 bp spacer-5′ *trn*V1 (39 bp)-600 bp intron-5′ *trn*V1 (35 bp) (Figure 1). In the *in vitro* system, the two divergent tRNA genes are transcribed and the primary transcription products are processed into a mature and correctly pseudouridylated $tRNA_2^{Met}$, and a partly processed $tRNA_1^{Val}$ which still contains the 600 nucleotide intron (Gruissem *et al.*, 1983b). We subcloned the *trn*M2 locus (including a 98-bp 5′ upstream region) as a 290-bp *Sau*3A-*Xba*I fragment into the vector pdX11. The resulting *trn*M2 plasmid, surprisingly, shows a 4- to 5-fold increase in transcription over the *trn*M2-*trn*V1 plasmid used above. This property is most likely a consequence of the physical separation of the *trn*M2 and *trn*V1 promoter regions.

Figure 2A shows the sequence of the 95 bp proximal to the *trn*M2 coding region and compares this sequence with the homologous sequences for the pea, tobacco, barley and maize chloroplast genomes. Two blocks of sequences (base pairs −3 to −16 and −37 to −85) show extensive (57%) sequence homology between species. The sequences immediately proximal to these conserved regions are randomised between species. Figure 2A also indicates the positions of the two blocks of conserved sequences that are most homologous to the prokaryotic

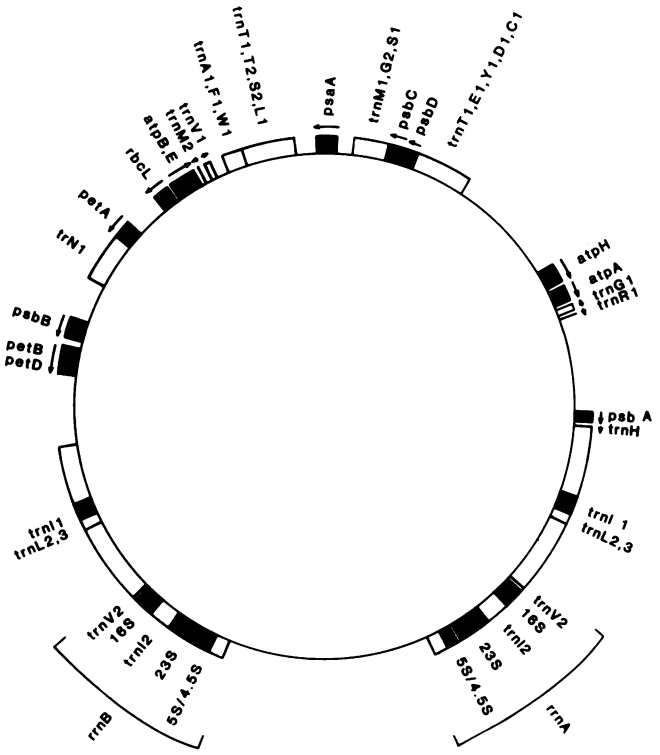

Fig. 1. Spinach chloroplast genome and transcription units. The spinach chloroplast DNA map was adapted from Driesel *et al.* (1979), Westhoff *et al.* (1983) and Gruissem *et al.* (1983b). The *trn*M2 transcription unit analysed in this report is located between the *atp*B,E and *trn*V1 transcriptional units. The polarity of *trn*M2 transcription is towards the 3′ end of the *atp*E locus (Gruissem *et al.*, 1983b). The *in vivo* $tRNA_2^{Met}$ transcript has been isolated from spinach chloroplast and sequenced, and the positions of base modifications have been determined (Pirtle *et al.*, 1981). Other chloroplast genes which have been transcribed *in vitro* are *trn*V1 (Gruissem *et al.*, 1983a), *trn*I1, which is located in the inverted repeat (Gruissem *et al.*, 1983b), *trn*I1, which is located in the inverted repeat the *atp*A and *trn*G1 transcriptional units (Gruissem and Zurawski, 1984). The *trn*H1 locus is co-transcribed with *psb*A *in vitro* (G. Zurawski and W. Gruissem, in preparation).

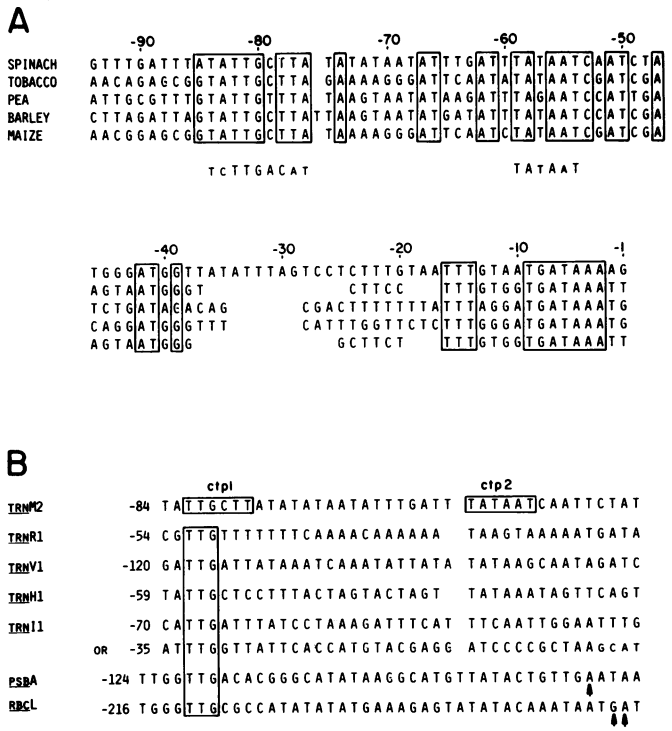

Fig. 2. Comparison of 5′-flanking regions of spinach chloroplast genes (**A**) The 5′ upstream DNA sequences of *trn*M2 from spinach, tobacco (Sugita and Sugiura, 1983), pea, barley (Zurawski and Clegg, 1984) and maize (Steinmetz *et al.*, 1983) were aligned for maximum homology. Regions with complete homology are shown in boxes. The pea *trn*M2 upstream DNA sequence has base substitutions at −79 and −57 relative to the spinach DNA sequence. The prokaryotic −35 and −10 consensus DNA sequence (Hawley and McClure, 1983) are aligned with *trn*M2 sequences (cpt1 and cpt2) that have partial or complete homology.
(**B**) Comparison of 5′ DNA sequences from spinach chloroplast genes which are transcribed in the chloroplast transcription system. Only those DNA sequences that have partial or complete homology to the *trn*M2 DNA sequences ctp1 and ctp2 are aligned. For *psb*A and *rbc*L *in vivo* transcription start sites have been determined by nuclease S1 mapping (arrows; Zurawski *et al.*, 1981, 1982).

−35 and −10 promoter elements (Pribnow, 1975; Rosenberg and Court, 1979; Siebenlist *et al.*, 1980). These two blocks are spaced (17 bp) similarly to their relative location in prokaryotic promoters (17 ± 1 bp; Hawley and McClure, 1983). To define which sequence elements (if any) within this conserved region are necessary for promoter function, we undertook a mutational analysis of this region.

Sequence requirements for trnM2 transcription

We constructed deletion mutants lacking sequences in the 5′ non-coding region of *trn*M2. The *Sau*3A site at base pair −98 of *trn*M2 provides a convenient start point for resection of the 5′ upstream region with *Bal*31 exonuclease. After repair of the resected ends, the truncated *trn*M2 DNA was recloned into the *Hinc*II-*Xba*I cut pdX11. The extent of deletion was determined by DNA sequence analysis, and the transcriptional properties of the deletion mutants were analysed in the chloroplast *in vitro* system. Figure 3 shows that removal of 5′ upstream DNA to base pair −83 (mutants 311, 37 and 310) has only a marginal effect on *in vitro trn*M2 transcription (>66% efficiency, Table I). Removal of sequences up to base pair −80 (mutants 34 and 314), however, results in a significant loss in transcriptional activity (<40% efficiency, Table I). Removal of sequences to base pair −76 (mutant 45) and beyond results in a drastic loss of transcriptional activity (<15% efficiency, Table I).

These data define the 5′ border of sequences required for efficient transcription of *trn*M2 as between base pairs −83 and −80. Since the chloroplast *in vitro* extract is efficient at correctly processing transcripts (Gruissem *et al.*, 1983b, Greenberg *et al.*, 1984b), there is a formal possibility that the mutational analysis is only measuring an effect on processing with subsequent destabilization of precursors and reduction in the assayed mature tRNA product. Control transcriptions with *Escherichai coli* RNA polymerase added to the *in vitro* system (pdX11 has a *lacZ* promoter upstream to the *trn*M2 insert) have shown that the deleted 5′ upstream sequences have no effect on $tRNA_2^{Met}$ processing (Gruissem *et al.*, 1983b). Deletions into the $tRNA_2^{Met}$ coding region do not generate stable, small RNA species (e.g., mutant 911) in the chloroplast extract or when *E. coli* RNA polymerase is added to the transcription system, although truncated tRNA molecules can be traced in a time course (data not shown). We interpret this as being due to lability of abnormal tRNAs in the chloroplast transcription extract.

Position of trnM2 promoter elements

It is possible that DNA sequences around base pair −80 of *trn*M2 are not the only requirement for transcription initiation by the chloroplast RNA polymerase. To investigate further the *trn*M2

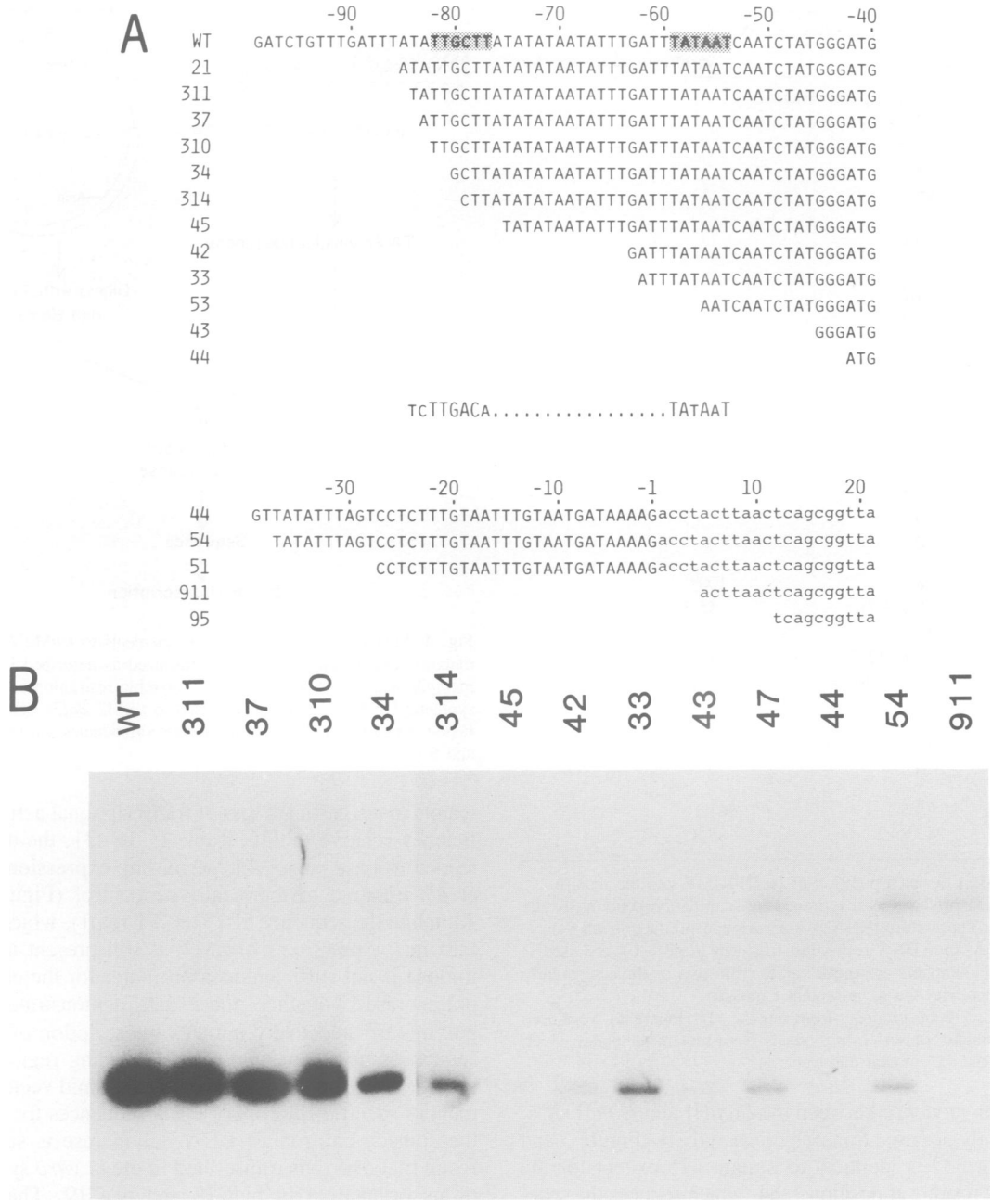

Fig. 3. Sequence requirement for *trn*M2 transcription. (**A**) DNA sequence of the *trn*M2 5′ upstream region and deletions obtained after resection with *Bal*31. The first 21 nucleotides of the *trn*M2 coding region are written in small letters. Blank areas indicate deleted DNA sequences which were replaced by pdX11 DNA sequences upstream from the *Sma*I restriction site. The consensus sequence for the prokaryotic −35 and −10 promoter elements are indicated and aligned for maximum homology with similar DNA sequences present in the 5′ upstream region of *trn*M2 (shaded boxes). (**B**) *In vitro* transcription of *trn*M2 deletion mutants in the spinach chloroplast extract. Transcription was routinely performed with equal copy numbers for the wid-type gene (*trn*M2 *Sau*3A-*Xba*I fragment in pdX11) and the deletion mutants in separate reactions. The incubation time was 60 min. No differences were observed in the relative efficiency of mutant or wild-type templates with varying amounts of DNA or different spinach chloroplast extracts (not shown). The autoradiogram shows the mature $tRNA_2^{Met}$ *in vitro* transcripts (76 nucleotides) from the mutant templates. The lanes are labeled with the numbers of the corresponding deletion mutants shown in (**A**). After autoradiography, the $tRNA_2^{Met}$ transcription products were excised from the gel and incorporation of [α-^{32}P]UMP was measured by scintillation counting. The transcription efficiencies of mutant templates relative to the wild-type gene are presented in Table I.

promoter, we undertook a systematic replacement and rearrangement of sequences between base pairs −82 and −45, which included the two blocks of conserved sequences that are most homologous to the prokaryotic −35 and −10 promoter elements. These *in vitro* manipulations took advantage of unique *Eco*RI and *Bam*HI restriction sites, respectively 19 bp and 9 bp, proximal to the *trn*M2 deletion end points in the *Bal*31-derived mutant plasmids (Figure 4). Complementary synthetic DNA with *Eco*RI and *Bam*HI ends were cloned into selected deletion mutant plasmids and the resulting constructs were sequenced and examined for their transcriptional activity. With synthetic DNAs encoding various amounts of 5′ upstream DNA deleted in the *Bal*31 mutants, we were able to create arrangements in which sequences were added, changed, or deleted with respect to the starting *trn*M2 plasmid. Mutant 425 has base substitutions of T to G, base pair −70; A to T, −69; T to C, −68; A to C, −67; T to G, −66 and T to C, −64, but is otherwise comparable with deletion mutant 310. These substitutions, which arise from

Table I. Relative transcription efficiencies of *trn*M2 5′ upstream mutant templates

Mutants	Transcription efficiency (% of wild-type control)[a]	
311	81	
37	66	
310	73	
34	38.9	
314	20	
45	4.2	
42	5.1	
33	14.5	
53	5.1	
43	4.3	
47	10.3	
44	1.9	
54	9.2	
51	4.8	
911	0	
95	0	
425	75	100[b]
332 (336)	35 (33.7)	55.5
451	30.7	
531 (532)	10.8 (12.2)	17.1
431 (432)	4.5 (6.8)	7.1
433	32	
514	58	
9111	0	
426	38.5	61.1
429	47.8	85.8

[a] Percent values reflect the incorporation of [α-^{32}P]UMP into the mature $tRNA_2^{Met}$ transcription products as determined by scintillation counting of the excised RNA band. The mature $tRNA_2^{Met}$ transcription products from the 290-bp *trn*M2 *Sau*3A-*Xba*I DNA restriction fragment in pdX11 were used as wild-type control. Numbers are mean values from two or three separate transcription reactions with the same mutant templates.

[b] The $tRNA_2^{Met}$ transcription products from mutant 425 (Figure 5) were used as a control to quantitate transcription products from mutant templates which were derived from the 425 mutant construct.

the pdX11 vector sequence between the *Bam*HI and *Hinc*II sites, do not significantly alter the transcriptional activity (Figure 5 and Table I). Mutant 332 is identical to mutant 425 except for the deletion of the C residue at position −64, which reduces the spacing of *trn*M2 sequences homologous to the prokaryotic consensus sequences from 17 bp to 16 bp. Interestingly, this mutant only supports transcription to 35% relative to the parental *trn*M2 plasmid or to 50% relative to 425 (Figure 5), thus suggesting the requirement of relative spacing of DNA sequences critical for transcription *in vitro*. This conclusion is supported by results from transcription of mutant 451, which has an 11-bp insertion at −75 (or relative to 425 has a deletion of base pair −64 and a 12-bp insertion at −64), and which has a reduced transcriptional activity (Table I). This construction generates the sequence 5′ TATAAT (cpt2) at a 14-bp spacing relative to the sequence 5′ TTGCTT (cpt1). The transcription efficiency of mutant 451 is comparable with mutant 332.

To test if sequences downstream from base pair −59, including the sequence 5′ TATAAT (cpt2), which is homologous to the prokaryotic −10 region (Pribnow, 1975), have any function for the *in vitro* transcription of *trn*M2, we constructed mutants in which part or all of this sequence was deleted (Figure 5). Mutant 531 has a deletion from −64 to −56 relative to 425, which

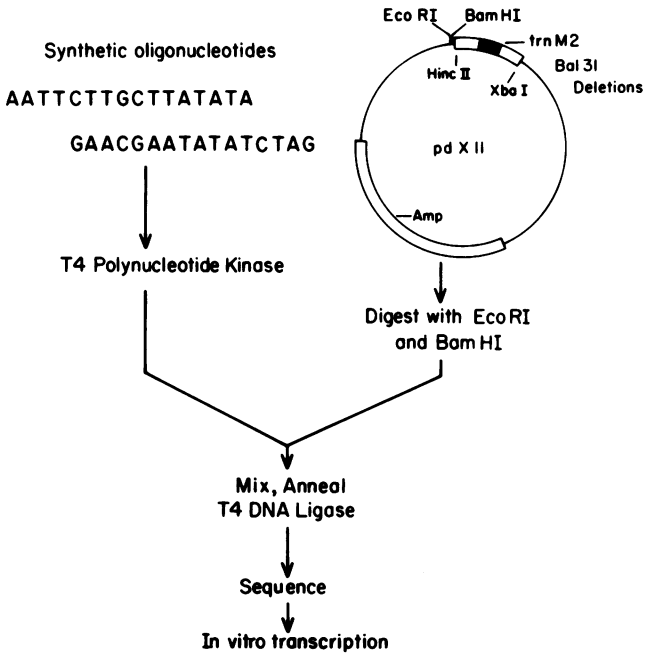

Fig. 4. Fusion of synthetic promoter elements to *trn*M2 *Bal*31 deletion mutants. Oligonucleotides were synthesized as described in Materials and methods with *Eco*RI and *Bam*HI compatible restriction enzyme ends. The synthetic DNA fragments were fused to *trn*M2 *Bal*31 deletion mutants. The fusion constructs and their transcription efficiencies are shown in Figures 5 and 6.

results in a significant loss of transcriptional activity (<20% efficiency relative to 425, Table I). In 431, the deletion was extended to base pair −45, permitting expression of *trn*M2 only at 5% relative to the wild-type control (Figure 5, Table I). Although the sequence 5′ TTGCTT (ctp1), which is required for maximal expression of *trn*M2, is still present in both mutants, it alone is not sufficient as a promoter for the chloroplast RNA polymerase. Together, these data demonstrate that the RNA polymerase selectively initiates transcription of $tRNA_2^{Met}$ from sequences upstream of the *trn*M2 coding region and not from sequences located elsewhere in the plasmid vector. The requirement of specific chloroplast DNA sequences for transcription by the spinach chloroplast RNA polymerase is supported by the result that *trn*M2 is transcribed in the *in vitro* system regardless of its orientation in pUC18 and pUC19. These experiments therefore delimitate the 5′ boundaries of at least two DNA regions required for *in vitro* transcription of *trn*M2, and implicate that sequences between −45 and −60 are essential for efficient expression.

In a first attempt to establish the 3′ boundary of 5′ DNA sequences essential for *trn*M2 expression, we synthesized complementary oligonucleotides encompassing the 5′ upstream DNA sequence from −41 to −82 (Figure 6). In mutant 433, the ligation of the synthetic DNA fragment to deletion mutant 43 results in a 10-bp insertion at −45. The insertion at this position reduces the transcription efficiency to 32% relative to the wild-type parental plasmid. Mutant 514 has a deletion (relative to the parental plasmid) of base pairs −27 to −35 and substitutions A to C, base pair −28; A to G, −30; T to C, −31 and T to C, −32. This plasmid is still an efficient template for transcription of *trn*M2 (58% relative to the wild-type control). No $tRNA_2^{Met}$ transcripts are made from mutant 9111, in which parental sequences between −41 and +5 are replaced with CCGTC. This is consistent with transcription results obtained from mutant 911.

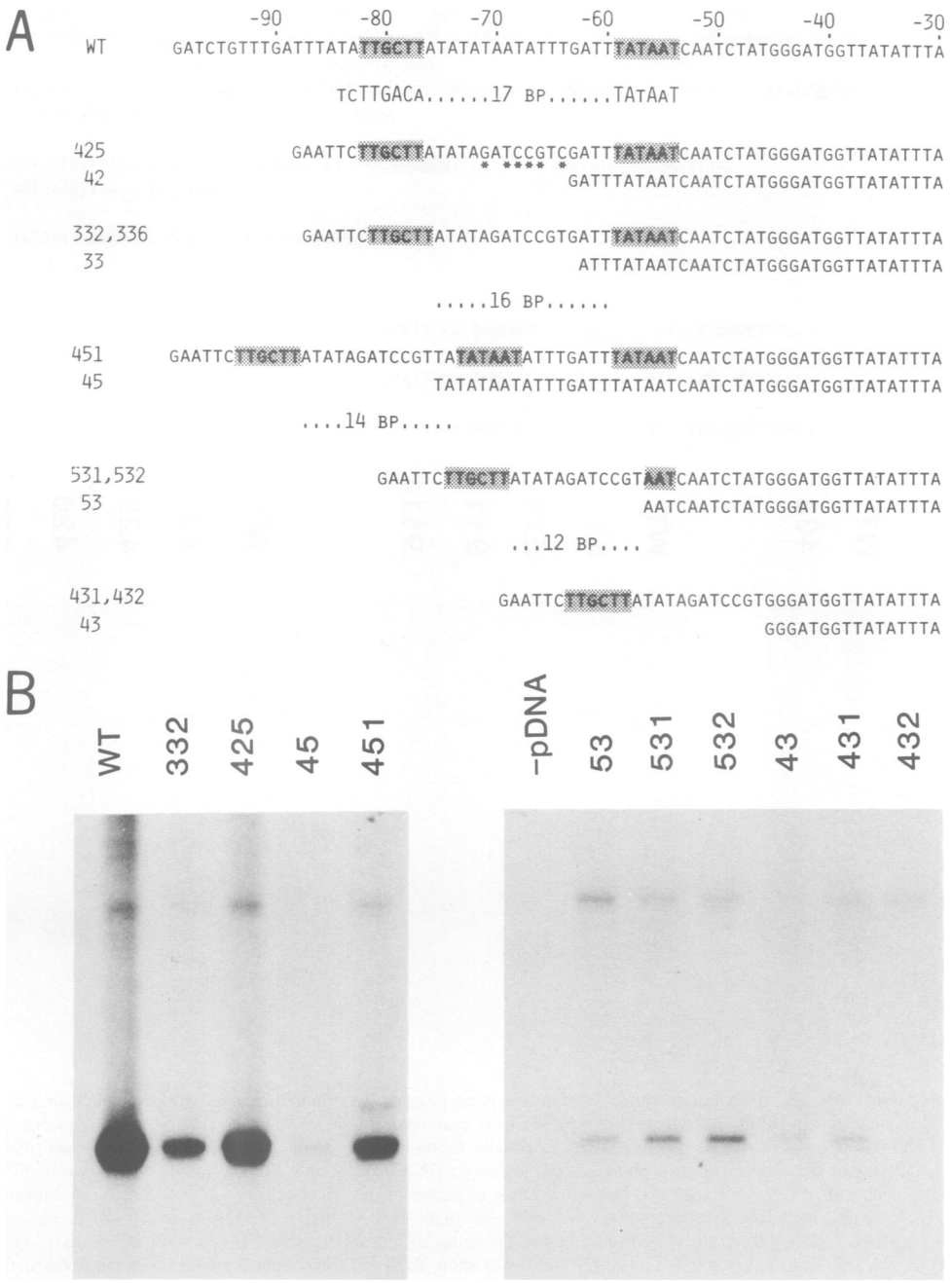

Fig. 5. Structure of analysis of *trn*M2 mutants that have a significant effect on transcription efficiency. (**A**) For the construction of 5′ upstream mutants, *trn*M2 deletion mutants 42, 33, 45 and 53 were digested with *Bam*HI and *Eco*RI and ligated to complementary oligonucleotides, resulting in the DNA insertion fragment 5′ GAATTCTTGCTTATATAGATCCGT, as described in Figure 4. Mutant pairs 332 and 336, 531 and 532, 431 and 432 are independent constructs with the identical sequence and a similar *in vitro* transcription efficiency (Table I). The *trn*M2 5′ DNA upstream sequence from −30 to the *Sau*3A restriction site at −98 is shown as the wild-type sequence (WT). The 5′ upstream DNA sequence of the respective *Bal*31 deletion mutants is indicated below the DNA sequence that resulted from fusion of the complementary oligonucleotides to these mutants. (**B**) *In vitro* transcription of *trn*M2 mutant constructs. Form I plasmid DNAs (60 μg/ml) were transcribed in the spinach chloroplast extract as described in Materials and methods. The incubation time was 90 min. Lane labeled WT shows the mature $tRNA_2^{Met}$ transcript (76 nucleotides) from the *trn*M2 *Sau*3A-*Xba*I DNA restriction fragment pdX11 (wild-type). Lanes 45, 53 and 43 are $tRNA_2^{Met}$ transcription products from the respective *Bal*31 deletion mutant templates (Figure 3). The other lanes are labeled with the numbers of the mutant constructs shown in (**A**). The relative transcription efficiencies for mutant constructs are listed in Table I.

Together these data implicate that the synthetic DNA fragment most likely contains the DNA sequences which can serve as a promoter in the *in vitro* transcription system. It is possible that DNA sequence insertions, deletions and substitutions downstream from −40 affect the transcription initiation site for the chloroplast RNA polymerase. We cannot disregard, however, a control function of this region for the rate of *trn*M2 transcription.

trnM2 promoter down-mutants

Since the *Bal*31 analysis implicated base pairs −80 to −83 as being the 5′ border of sequence requirement for the *trn*M2 promoter, we initiated experiments to evaluate the function of single nucleotides in this vicinity for promoter strength and transcription initiation frequency. Figure 6 shows the DNA sequence and transcription results for two plasmids with single base substitu-

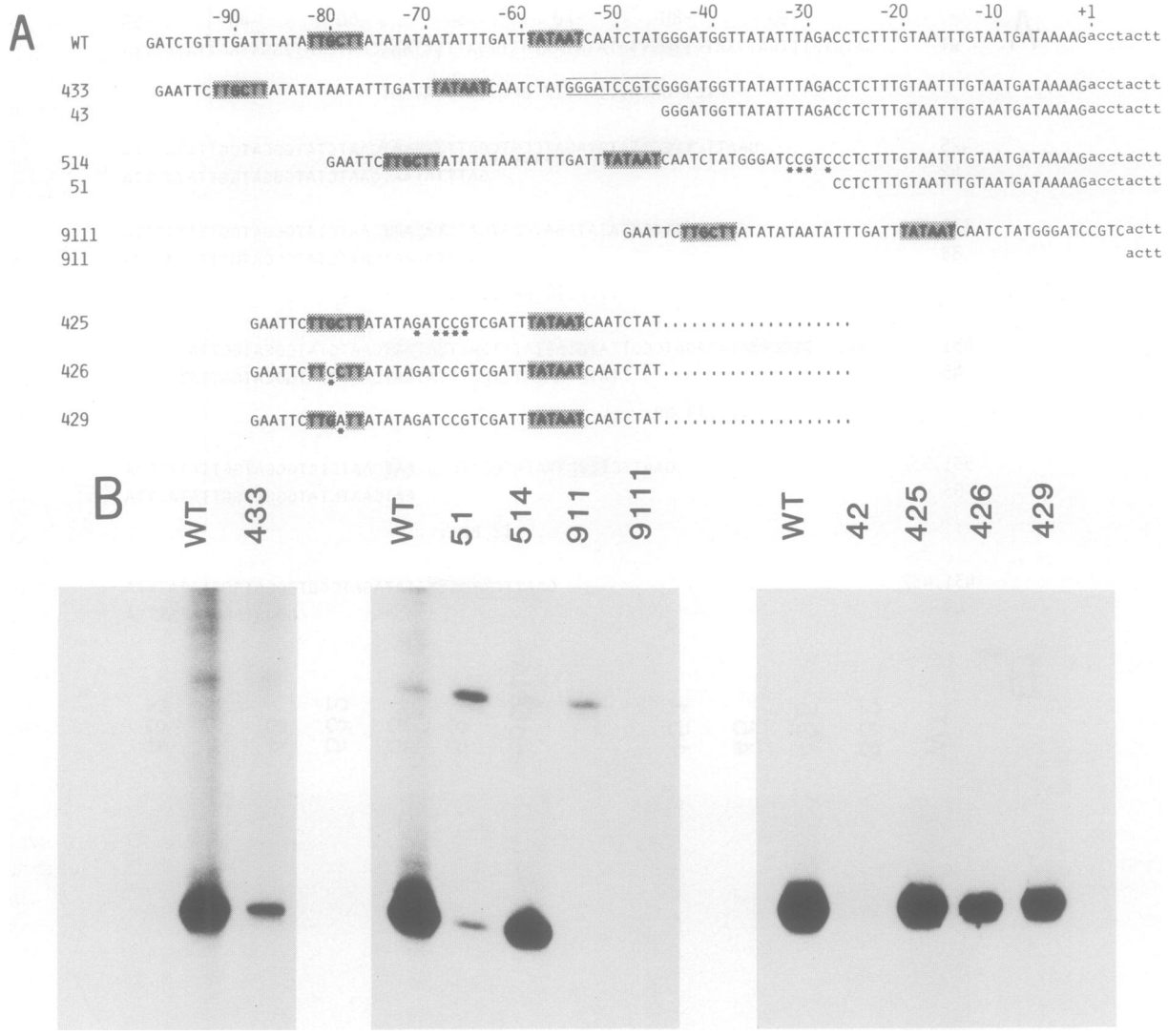

Fig. 6. Structure and analysis of *trn*M2 5′ upstream insertion, deletion and point mutants. **(A)** *Bam*HI/*Eco*RI-digested deletion mutant plasmid DNAs were ligated to complementary oligonucleotides with *Eco*RI/*Bam*HI compatible ends encompassing the *trn*M2 wild-type 5′ DNA sequence from −41 to −82. Oligonucleotide synthesis and construction of mutant plasmid DNAs essentially followed the procedure described in Materials and methods. The *trn*M2 5′ DNA upstream sequence and part of the coding region is shown as the wild-type DNA sequence (WT). The *trn*M2 5′ upstream DNA sequence of the respective *Bal*31 deletion mutants (43, 51, 911) is indicated below the DNA sequence of the oligonucleotide fusion constructs. Mutants 426 and 429 are similar to mutant 425 (Figure 5), but have base substitutions at base pairs −80 (426, G to C) and −79 (429, C to A). **(B)** *In vitro* transcription of *trn*M2 mutant constructs. Form I plasmid DNAs (60 μg/ml) were transcribed in the spinach chloroplast extract as described in Figure 3. The incubation time was 90 min. Lanes WT, 433; WT, 51, 514, 911, 9111; and WT, 42, 425, 426, 429 show $tRNA^{Met}$ transcription products (76 nucleotides) from three different sets of experiments, respectively.

tions at −80 (mutant 426, G to C) and −79 (mutant 429, C to A). Compared with their parent plasmid (mutant 425, 75% transcription efficiency), the single point mutations reduced transcription efficiency to 39% and 48%, respectively (Table I). The pronounced effect suggests that the substituted nulceotides in the tested positions seem to be particularly important for transcription promotion, since both substitutions result in down-mutations. Most interestingly, the base substitution at −79 (C to A) results in a DNA sequence more closely related to the prokaryotic −35 (TTGACT) consensus sequence (Hawley and McClure, 1983), but does not result in a promoter-up mutation for the chloroplast RNA polymerase. A possible divergence of promoter sequence requirement from the prokaryotic −35 promoter element is also implicated by the finding that the *lac*Z promoter in pUC18 and pUC19 (Vieira and Messing, 1982) and *lac*UV5 promoter (Sienbelist *et al.*, 1980) are only poor promoter regions for the spinach chloroplast RNA polymerase.

Function of trnM2 upstream sequences

The critical question raised by the results reported here is whether the 'prokaryotic-type' sequence requirement for maximal transcription efficiency is a unique property of spinach *trn*M2. This is argued against by our finding that the spinach chloroplast RNA polymerase transcribes *trn*M2 from tobacco (Sugita and Sugiura, 1983) and barley (Zurawski and Clegg, 1984) at the same rate *in vitro* (not shown), suggesting a conserved promoter requirement for *trn*M2 from monocotyledons and dicotyledons. Considering the poor sequence conservation in the upstream region from −17 to −40 between spinach and tobacco (Figure 2A), it also strengthens our conlcusion that DNA sequences essential for promoter function are contained in the synthetic DNA fragment encompassing the spinach chloroplast *trn*M2 upstream region from −41 to −82. Interestingly, no $tRNA_2^{Met}$ transcripts have been detected from pea *trn*M2 in the spinach chloroplast transcription extract. This could be the consequence of base

substitutions which occur in the regions essential for *trn*M2 transcription (C to T, base pair −79; T to G, base pair −57, Figure 2A), implicating a different sequence requirement for the pea RNA polymerase which might have resulted from major rearrangements and extreme base substitutions in legumes (Palmer and Thompson, 1982). Major promoter elements are also present in synthetic DNA fragments encompassing the 5′ upstream regions of *psb*A and *rbc*L shown in Figure 2B. When fused to *trn*M2 deletion mutant 51 the resulting plasmid DNA supports the transcription of $tRNA_2^{Met}$ (G. Zurawaski and W. Gruissem, in preparation). Since different RNA polymerase activities have been identified in chloroplasts, it is unknown if the RNA polymerase responsible for *trn*M2 transcription also recognizes these promoter elements.

The comparison of 5′ DNA sequences from chloroplast tRNA transcription units *trn*M2, *trn*R1, *trn*V1, *trn*H1 and *trn*I1, as well as *psb*A and *rbc*L (Figure 2B; for location see Figure 1) does not allow the prediction of a 'consensus sequence' or the generalization of the *trn*M2 promoter concept. Instead, the difference in 5′ DNA sequence organization could be the explanation for the different transcription rates for these genes *in vivo* and *in vitro*. We have previously reported the transcription of *trn*I1, *trn*H1 and *trn*R1 and compared it with the transcription rate of *trn*M2 (Gruissem *et al.*, 1983b; Gruissem and Zurawski, 1984). We found that the transcription efficiency of *trn*I1 is only 20% relative to *trn*M2 *in vitro* and *in vivo*, and that *trn*R1 is transcribed only at a several-fold lower rate. In addition, we can delete the entire 5′ upstream DNA sequence of *trn*R1 and still retain full transcriptional activity (C. Elsner-Menzel and W. Gruissem, unpublished results), suggesting an entirely different transcription mechanism for this gene. Both $tRNA_1^{Ile}$ and $tRNA_1^{Arg}$ are minor isoacceptors with infrequent codon usage in chloroplast mRNAs [0% and 0.56% for $tRNA_1^{Ile}$ (ATG), 0.84% and 0.84% for $tRNA_1^{Arg}$ (AGA,AGG)] in *psb*A and *rbc*L mRNAs, respectively. It is possible, therefore, that DNA sequences other than essential sequences for *trn*M2 transcription function as effective mechanisms to control the transcription rate of different chloroplast tRNA isoacceptors. A different control mechanism might also operate for the transcription of *trn*H1. We have previously reported that *trn*H1 is not transcribed in a plasmid which lacks the 5′ upstream region of *psb*A (Gruissem *et al.*, 1983b; Gruissem and Zurawski, 1984). Although a good homology of *trn*H1 5′ DNA sequences exists with essential *trn*M2 promoter sequences (Figure 2B), this apparently is not sufficient for the expression of this gene. Co-transcription of the *psb*A-*trn*H1 transcription unit occurs *in vitro* when the *psb*A 5′ DNA sequence shown in Figure 2B is present in the plasmid (Gruissem and Zurawski, 1984). It has been shown for *psb*A in mustard that expression of this gene *in vitro* requires 5′ upstream DNA sequences. Similar to the promoter requirements described for *trn*M2 in this report, it appears that a −35 promoter element is most critical for efficient transcription of mustard *psb*A (Link, 1984).

Conclusions

We have used a chloroplast transcription system to characterize the DNA sequence requirement for transcription of a spinach chloroplast tRNA transcription unit by the chloroplast RNA polymerase. The experiments described here are of interest for several reasons. Of primary interest is the demonstration that the spinach chloroplast extract can be used to define promoter regions recognized by the chloroplast RNA polymerase. Since we find certain transcription modes conserved *in vitro*, it is most likely that similar promoter requirements exist *in vivo*. The observation that *trn*M2 from evolutionary distinct plant species are transcribed by the spinach chloroplast RNA polymerase must reflect significant conservation of sequences required for transcription. The mutational analysis indicates that the spinach chloroplast *trn*M2 82 bp 5′ upstream region can tolerate considerable sequence flexibility and yet retain significant transcriptional activity. With internal deletions we only found gross reduction in template efficiency in two mutants (531 with an 8-bp deletion and 431 with a 19-bp deletion). These two mutants may remove sequences critical to promoter function or change relative spacing between critical promoter sequences. Interestingly, these mutants lack all or part of the 5′ TATAAT (cpt2) sequence that is homologous to the prokaryotic −10 element. The mutants that have less drastic effects on template activity are useful in defining those 5′ upstream sequences that are not critical to promoter function. Deletion and replacement of sequences from −27 to −35, a region that corresponds to the most poorly conserved *trn*M2 upstream sequence, has little effect on template activities. Substitutions of nucleotides in the −65 to −70 region also has a minimal effect on transcription efficiency. Conversely, changes in the length of this region always result in a significant loss of transcription. These latter changes are of interest, since they occur between the two regions homologous to the prokaryotic −35 and −10 sequences. Thus, a DNA sequence requirement for transcription of the spinach chloroplast *trn*M2 locus by the homologous RNA polymerase emerges which resembles the prokaryotic promoter organization. A more detailed mutational analysis of this region should help to establish the evolutionary relationship of prokaryotic and chloroplast promoter regions. Furthermore, as the promoter requirements for several chloroplast genes can now be determined by the transcription of mutant templates of *trn*M2/promoter fusion *in vitro*, it will be possible to develop a general model for chloroplast promoter function.

Materials and methods

Plasmid DNA

Plasmid pSocE55 contains the spinach chloroplast *trn*M2 and *trn*V1 loci as a 1.65-kb *Eco*RI fragment in pBR322. A 290-bp *Sau*3A-*Xba*I fragment containing *trn*M2 and 98 bp of 5′ upstream DNA sequence was subcloned into the *Bam*HI-*Xba*I restriction sites of pdX11. Plasmid pdX11 is a derivative of pUC8 with additional *Xba*I and *Bgl*II restriction enzyme sites between the *Pst*I and *Hind*III restriction enzyme sites in the polylinker region (M. Benedik, personal communication). The *trn*M2 subclone was used to generate exonuclease *Bal*31 deletion mutants in the 5′ upstream region. Plasmid DNA was linearized at the *Sma*I site at position −105 upstream from the 5′ end of the *trn*M2 coding region. 100 μg of the *Sma*I-digested plasmid DNA was then treated with the double-stranded exonuclease *Bal*31 for various times. Under the chosen conditions up to 150 bp were resected from the *Sma*I restriction site. The *Bal*31-treated DNA was then pooled and incubated with T4 DNA polymerase to increase the concentration of blunt ends. After digestion with *Xba*I, fragments were separated on a preparative agarose gel, and 12 gel slices were excised containing DNA fragments ranging from 150 to 290 bp. After elution from the gel and column purification, DNA fragments from each gel section were cloned into *Hin*cII- and *Xba*I-digested pdX11, and transformed into JM101. Inserts containing recombinant plasmids were identified by their white color reaction on X-gal plates (Messing, 1983), and the extent of deletions was monitored by DNA sequencing (Maxam and Gilbert, 1980).

Plasmid DNAs were isolated by a modified cleared lysate procedure (Clewell, 1972) or the alkaline-SDS method (Birnboim and Doly, 1979). The crude plasmid DNA fractions were treated with RNase and proteinase K prior to centrifugation. Supercoiled DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. Purified plasmids were typically >70% form I DNA.

Construction of trnM2 promoter mutants

Complementary oligonucleotides of varying length were synthesized with *Eco*RI/*Bam*HI compatible ends, which either encompassed the wild-type DNA

segments in the *trn*M2 5′ region from base pairs −72 to −82, base pairs −41 to −82 or contained base substitutions as described in the text. Oligonucleotides were synthesized with the Applied Biosystems synthesizer (solid support/solid phase phosphoamidazide method, Matteucci and Caruthers, 1981), purified by electrophoresis on a 20% polyacrylamide-8 M urea gel and localized by u.v.shadowing. After elution from the gel, purification by DEAE52 chromatography and drying, the oligonucleotide concentration was adjusted to 20 $O.D._{260}$/ml. 1 μl of purified oligonucleotides (120−150 nmol) was used for the phosphorylation of 5′-OH ends in 15 μl reactions containing ATP and T4 polynucleotide kinase. pdX11 DNAs (0.5 μg) with selected *trn*M2 5′ deletions were digested with *Eco*RI and *Bam*HI. 10−20 nmol of oligonucleotides were mixed with the restriction enzyme-digested DNA and ligated with T4 DNA ligase at 25°C for 4 h. Competent JM101 cells were transformed with 10 μl of the ligation mixture (Messing, 1983), and ampicillin-resistant colonies were screened for the insertion of the synthetic DNA fragments by hybridization. Colonies were replica plated on Whatman 540 paper and the cells were lysed with 0.5 M NaOH, 1.5 M NaCl. After neutralization and baking for 2 h at 80°C the filters were prehybridised in 6 x SSC (20 x SSC: 3 M NaCl, 0.3 M Na-citrate, pH 7), 20% formamide, 0.1% SDS and 100 μg/ml tRNA for 1 h at 42°C. Coomassie stain was added to visualize the location of colonies. Oligonucleotides to use as hybridisation probes were labeled with [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase for 30 min at 37°C and purified by Sephadex G50 chromatography. The filters were then hybridised with the respective oligonucleotides for 2 h at 42°C. After hybridisation filters were washed twice for 5 min at 25°C in 1 x SSPE (20 x SSPE: 3 M NaCl, 0.23 M NaH_2PO_4, 0.023 M EDTA, pH 7.4), 0.1% SDS, and once for 5 min at 37°C in 0.1 x SSPE. Hybridisation to single colonies was usually detected after a 4−8 h exposure. Plasmid DNAs from positive transformants were isolated and sequenced to verify the correct constructions and ligation boundaries.

Chloroplast transcription extract

Spinacea oleracea was grown hydroponically on one-half strength Hoagland's solution (Hoagland and Arnon, 1939) under greenhouse conditions. Fully developed spinach leaves (5−10 cm length) were used for the isolation of intact chloroplasts (Gruissem, 1984). Intact chloroplast were isolated after centrifugation through percol gradients as described (Gruissem *et al.*, 1983b). The chloroplast extract was prepared according to a described procedure (Gruissem *et al.*, 1982) with several modifications (Gruissem *et al.*, 1983b; Gruissem, 1984). Briefly, chloroplasts were lysed in a hypotonic buffer and stromal proteins were extracted with 0.5 M ammonium sulfate. The membrane material, including a DNA-bound RNA polymerase activity (transcriptionally active chromosome, TAC; Gruissem *et al.*, 1983b, Gruissem and Zurawski, 1984), was removed by centrifugation. The supernatant fraction was then subjected to DEAE column chromatography, and proteins from the DEAE column fraction were precipitated with ammonium sulfate. After resuspension of the protein pellet and dialysis, the extract was used for transcription experiments.

In vitro transcription

Plasmid DNAs (predominately form I; 60 μg/ml) were incubated under standard conditions (Gruissem *et al.*, 1983b; Gruissem, 1984). The relative amount of form I DNA was monitored for each plasmid construct and was typically >70%. The *in vitro* reaction mixture contained 10 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfuric acid (Hepes), 5 mM Tris-HCl, pH 7.9 (20°C), 0.1 mM EDTA, 10 mM $MgCl_2$, 1.5 mM dithiothreitol (DTT), 40 mM KCl and 10% glycerol. The concentration of nucleotides was 500 μM for ATP, CTP and GTP, 25 μM UTP and 10 μCi [α-^{32}P]UTP (410 Ci/mmol). Spinach chloroplast extract (12.5 μl) was added to reactions of 25 μl final volume. The reaction mixtures were incubated at 25°C for 60 or 90 min (see Figure legends). Transcription was terminated by addition of proteinase K/SDS at 37°C for 15 min and subsequent phenol/chloroform extraction. After ethanol precipitation, transcription products were resuspended in 98% formamide, 10 mM piperazine-N,N′-bis[2-ethanesulfonic acid], 0.2% SDS, and separated on 10% polyacrylamide-8 M urea gels. After localization of bands for the $tRNA_2^{Met}$ transcripts on the X-ray film they were excised from the gel and incorporation of [α-^{32}P]UMP into mature $tRNA_2^{Met}$ transcription products was determined by scintillation counting.

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